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Purification and characterization of the cystinyl bond cleaving yeast aminopeptidase yscXVI

Ursula Tisljar and Dieter H. Wolf

Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart, Germany

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Aminopeptidase yscXVI was purified from the yeast *Saccharomyces cerevisiae*. By SDS-PAGE the enzyme has a molecular weight of 45,000 Da, and in chromatofocusing, elution was observed at pH 6.2. The synthetic substrate cystinyl-4-nitroanilide (K_m 22.5 μM, V_{max} 12.9 mU/mg) is cleaved most efficiently in the pH range 7–8. Besides cleaving this standard substrate, aminopeptidase yscXVI acts on several other 4-nitroanilide substrates with unsubstituted N-terminal L-amino acids. Highest hydrolysis rate was measured with Lys-4-nitroanilide and Leu-4-nitroanilide. The activity of aminopeptidase yscXVI is abolished by chelating agents and restored by Zn^{2+}, Mn^{2+} and Co^{2+} ions. Bestatin and amastatin are both strong inhibitors of the enzyme, with K_i values of 0.53 μM and 0.93 μM, respectively. Aminopeptidase yscXVI is detectable in the logarithmic growth phase, stationary phase, and in starved cultures of yeast.

Protease; Yeast aminopeptidase

1. INTRODUCTION

The yeast *Saccharomyces cerevisiae* is an excellent tool to study proteolysis and its catalysts in eukaryotic cells because it is easily amenable to biochemical and genetic manipulations (for reviews see [1–5]). The function of yeast proteinases ranges from limited proteolysis to unlimited protein degradation. Limited proteolysis includes pheromone processing catalyzed by the *KEX2*- and *KEX1*-encoded proteinase yscF and carboxypeptidase yscα, respectively, and the *STE13*-encoded dipeptidyl-aminopeptidase yscIV (DPAPA) [1–4] and processing of mitochondrial proteins by the *MAS*-encoded processing protease [6]. The unlimited protein degradation takes place in the lysosomal-like yeast vacuole [1–4] and in the cytoplasm [5]. Proteinases yscA and yscB [1–4,7–9], carboxypeptidase yscY and yscS [1–4,10,11], aminopeptidase yscI and dipeptidylaminopeptidase yscV (DPAPB) belong to the extensively studied vacuolar peptidases [1–4]. In the cytoplasm and the proteasome, proteinase yscE is responsible for the degradation of ubiquitinated proteins [5,12–15] and proteinase yscD is involved in the degradation of smaller peptides [16,17].

Correspondence address: U. Tisljar, Institut für Pharmazeutische Biologie, Albert-Ludwigs-Universität, Schänzlestr. 1, 7800 Freiburg, Germany. Fax: (49) (761) 203 2767.

Abbreviations: Ac, acetyl; Bz, benzoyl; $(Cys\text{-}NH\text{-}Ph)_2$, cystinyl-4-nitroanilide; E-64, L-3-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butane; NH-Ph, 4-nitroanilide; PMSF, phenylmethyl-sulfonyl fluoride; Suc, succinyl; Z, benzyloxycarbonyl.

While many of the endopeptidases are well characterized less detailed information is available about the numerous yeast aminopeptidases. Besides the extensively studied methionine aminopeptidase [18–20], a multitude of aminopeptidase activities was discovered by fractionation of crude yeast extracts on ion-exchange and gel filtration resins [21]. The activities were named aminopeptidase yscI to yscXV and aminopeptidase P [1–3,21]. So far, only two of those activities, aminopeptidase yscI and yscII, have been studied thoroughly [22–27]. In addition Trumbly and Bradly identified four different aminopeptidases in yeast which they called LAP I to LAP IV [28]. It seems that aminopeptidase yscI is identical to LAP IV, and aminopeptidase yscII is identical to LAP I. Another aminopeptidase, known as aminopeptidase yscCo is only detectable in the presence of Co^{2+} ions [29].

In a previous study [30] activities were discovered that hydrolyze cysteinyl bonds in a 4-nitroanilide substrate. In this investigation we purified and characterized the corresponding enzyme and named it aminopeptidase yscXVI.

2. EXPERIMENTAL

2.1. *Yeast strains and growth conditions*

Aminopeptidase yscXVI was purified from a stationary phase culture of the strain FABYSD-17C (*MATa kex2-1 pra1-1 prb1-1 prc1-1 cps1-3 prd1 ade his lys*) [31]. Cells were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 50 μg/ml adenine and uracil at 30°C for 24 h. Enzyme activity was assayed in cells that were harvested in the logarithmic growth phase, in the stationary growth phase, or after starvation, and in a stationary growth phase culture of strain YHH26 (*ape2-1 leu2-3, 112 ura3Δ5*).

2.2. *Enzyme assay*

Unless otherwise stated 10–100 μl of sample were mixed with 5 μl (Cys-NH-Ph)$_2$ (10 mM stock solution in dimethyl formamide) and 50 mM Tris-HCl, pH 7.2) to a total volume of 900 μl. After incubation at 30°C for 1 h the reaction was stopped with 100 μl of 1 M acetic acid and the A_{405} was recorded. A sample blank without substrate was included when crude yeast extract was assayed. In some experiments other 4-nitroanilide substrates were used similarly. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of 4-nitroaniline per min. Calculations are based on a molar absorption coefficient of 9,500 l/mol × cm for 4-nitroaniline at 405 nm.

2.3. *Protein assay*

The Bradford assay [32] was used with bovine serum albumin as standard.

2.4. *Preparation of crude yeast extracts and vacuoles*

To measure specific enzyme activity in different yeast strains and under different growth conditions, cells were grown in 15 ml YPD medium to the appropriate growth phase, and centrifuged at 2,000 × *g* for 4 min. The pellet was washed with distilled water, suspended in 200–300 μl 50 mM Tris-HCl, pH 7.2, and transferred to an Eppendorf tube. Glass beads (200–300 μl, 0.45 mm diameter) were added, the tubes were mixed vigorously in an IKA Vibrax VXR at 8°C for 10 min and then centrifuged at 10,000 × g at 8°C for 15 min. Enzyme activity and protein were assayed in the supernatant.

Vacuoles were prepared from a stationary culture of strain FABYSD-17C as previously described [28]. Enzyme activity was assayed immediately after preparation of the vacuoles.

2.5. *Enzyme purification*

All purification steps were done at 8°C. Cells (27–32 g) of 2 l cultures were harvested by centrifugation, washed with distilled water, resuspended in 1 vol. of buffer A (25 mM Tris-HCl, pH 7.5), and disintegrated by passing three times through a French pressure cell set at 20,000 psi. The crude extract was centrifuged at 10,000 × *g* for 30 min. After dialysis of the supernatant against 2 l of buffer A, the extract was applied for DEAE-Sephacel chromatography. Conditions were: bed size 30 ml, diameter of the column 1.6 cm, eluted with a linear gradient from 0 to 300 mM NaCl in buffer A, total gradient volume 140 ml, flow rate 0.7 ml/min, fraction size 2 ml. Two peaks of activity were detected with (Cys-NH-Ph)$_2$. Fractions of the early eluting peak were combined and used for further purification. After concentration over an Amicon PM10 membrane the sample was run on a column of 170 ml Sephacryl S-300 HR, diameter 1.6 cm, eluted with 50 mM Tris-HCl, 200 mM NaCl, pH 7.5, flow rate 0.5 ml/min, fraction size 2.5 ml. The fractions with the highest activity were combined, dialysed against 2 l of buffer B (25 mM Tris-HCl, pH 8.0) and run on a Mono Q HR5/5 FPLC column in several aliquots. The conditions were: linear gradient from 0 to 200 mM NaCl in buffer B, total gradient volume 30 ml, flow rate 1 ml, fraction size 0.5 ml. The active fractions were combined, dialysed against 2 l of buffer C (25 mM bis-Tris-HCl, pH 7.2) and applied for chromatofocusing on a column of 9 ml PBE 94 (Pharmacia), diameter 0.9 cm, equilibrated with buffer C. The column was eluted with polybuffer 74, 1:10 diluted with distilled water and adjusted to pH 4 with HCl. The flow rate was 0.5 ml/min and the fraction size was 2.5 ml. The active fractions were combined and dialysed against 2 l of 50 mM Tris-HCl, pH 7.2, and stored at 4°C until used for the characterization studies.

In some experiments fractions of the late eluting peak of the DEAE-Sephacel chromatography step were combined and further purified as described for the early eluting form of the enzyme.

2.6. *SDS-PAGE*

Purified enzyme was run under reducing conditions in a polyacrylamide gel (total acrylamide concentration 12%) as described by Laemmli [33]. The molecular mass was determined by comparison with the following standards (Sigma): triosephosphate isomerase (26.6 kDa), lactic dehydrogenase (36.5 kDa), fumarase (48.5 kDa), pyruvate kinase (58 kDa), fructose-6-phosphate kinase (84 kDa), and β-galactosidase (116 kDa). In the same PAGE system the enzyme was run without denaturation and reduction. One lane of the gel was stained for protein and the other lane was cut transversely into narrow strips for enzyme assays.

2.7. *Analytical gel chromatography*

The purified enzyme was run on a Superose 12 HR 10/30 FPLC column in 50 mM Tris-HCl, 200 mM NaCl, pH 7.5, at 0.25 ml/min. The fractions (0.25 ml) were assayed for enzyme activity. The column was calibrated with bovine serum albumin (67 kDa), ovalbumin (45 kDa), and chymotrypsin (25 kDa).

2.8. *Deglycosylation*

The purified enzyme (40 μg) was treated with *N*-glycosidase F (Boehringer-Mannheim) as recommended by the manufacturer, and run on SDS PAGE before and after treatment.

2.9. *Kinetic data*

For kinetic studies the hydrolysis rate of (Cys-NH-Ph)$_2$ was monitored continuously. K_m and K_i values were calculated by standard methods using the Enzfitter program (Elsevier/Biosoft, Cambridge, England). The apparent inhibition constants were corrected for the effect of substrate as previously described [34].

3. RESULTS

3.1. *Purification and physicochemical properties of aminopeptidase yscXVI*

To avoid degradation of the proteins in crude extracts by intrinsic yeast peptidases aminopeptidase yscXVI was purified from a yeast strain deficient in the activities of proteinase yscF, yscA, yscB, yscD, and carboxypeptidase yscY, and yscS. Results of the DEAE ion-exchange chromatography steps are illustrated in Fig. 1. Table I summarizes the purification of the early eluting form of aminopeptidase yscXVI. In the last step of purification, the chromatofocusing step, activity against (Cys-NH-Ph)$_2$ and Leu-NH-Ph co-eluted from the column. With SDS-PAGE we detected a single band of protein with a molecular mass of 45,000 Da (Fig. 2). One single peak of protein with corresponding enzyme activity was eluted from the analytical gel chromatogra-

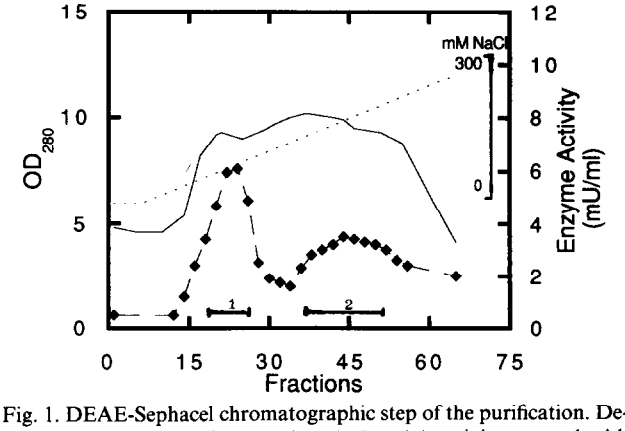

Fig. 1. DEAE-Sephacel chromatographic step of the purification. Details are described in the text. (——) A_{280}; (◆) activity assayed with (Cys-NH-Ph)$_2$, (---) NaCl concentration. (1) Early eluting form of the enzyme. (2) Late eluting form of the enzyme.

Table I

Aminopeptidase yscXVI was purified from 27 g yeast as described in section 2

Fraction	Protein (mg)	Activity (mU)	Specific activity (mU/mg)	Yield (%)
10,000 × *g* supernatant of crude extract	2,080	596	0.286	(100)
DEAE-Sephacel (early eluting form)	158	89	0.563	15
Sephacryl S-300 HR	54	74	1.370	12
Mono Q	8.6	39	4.530	6.5
Chromatofocusing	2.25	29	12.9	4.9

phy column (Fig. 3). The estimated molecular mass is 52,000 Da. In the non-denaturing polyacrylamide gel, protein staining and enzyme activity were localized at the same position (not shown). The results of the SDS-PAGE, gel filtration chromatography and non-denaturing polyacrylamide electrophoresis give evidence that the protein was purified to homogeneity. After treatment of aminopeptidase yscXVI with *N*-glycosidase F, no change of the molecular mass was observed. *N*-Glycosylation of the enzyme is therefore excluded. From the chromatofocusing step of the purification we estimate an isoelectric point of 6.2 for aminopeptidase yscXVI. A linear hydrolysis rate of $(Cys\text{-}NH\text{-}Ph)_2$ was observed at 30°C over a period of 1 h. No activity was measured in sodium acetate buffer at pH 4. In bis-Tris-HCl buffer, pH 6, about 1/3 of the maximal activity was assayed. The substrate was cleaved most efficiently in the pH range 7–8. No activity was detected in sodium carbonate buffer at pH 9. The enzyme is rather unstable. The purification yield was only 5% and after storage of the purified enzyme at 4°C, 62% and 19% of the initial activity was assayed after 4 and 11 days, respectively.

When the late eluting peak of DEAE-Sephacel chromatography (Fig. 1) was further purified, the activity was eluted at the same position as the early eluting form in Sephacryl S-300 HR gel chromatography, Mono Q ion-exchange chromatography and chromatofocusing. The purified late eluting form of the enzyme was inhibited by EDTA, and hydrolyzed the same substrates as the early eluting form of aminopeptidase yscXVI (details not given). We do not know whether two slightly different forms of the enzyme exist in vivo or whether the two forms represent artefacts generated during the disintegration of the yeast cells.

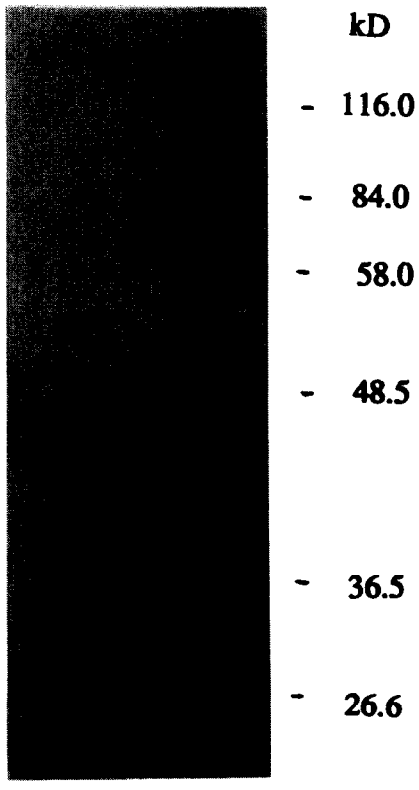

Fig. 2. SDS-PAGE. 17 μg of the purified enzyme was run reduced in a SDS-polyacrylamide gel alongside standards, as described in section 2.

3.2. *Inhibition studies*

Enzyme activity was not affected by inhibitors of

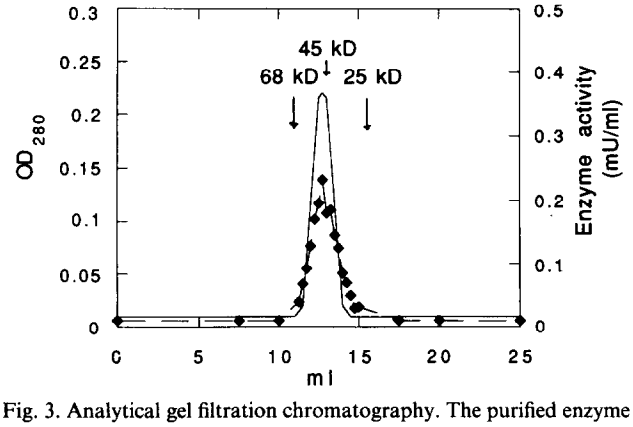

Fig. 3. Analytical gel filtration chromatography. The purified enzyme was loaded on a Superose 12 column and eluted as described in section 2. The column was calibrated with bovine serum albumin, ovalbumin, and chymotrypsin.

Table II

Hydrolysis of (Cys-NH-Ph)$_2$ was assayed in the presence of various inhibitors

Inhibitor	Concentration	% of control
PMSF	1 mM	97
E-64	10 μM	116
N-Ethylmaleimide	1 mM	103
Pepstatin	10 μg/ml	116
EDTA	10 mM	3
1,10-Phenanthroline	1 mM	17
Phosphoramidon	0.1 mM	102
Puromycin	0.1 mM	93
Bestatin	K_i 0.54 μM	
Amastatin	K_i 0.93 μM	

The preincubation time was 20 min at 30°C for all inhibitors except for EDTA, bestatin and amastatin. EDTA preincubation time was 24 h at 4°C. Inhibition by bestatin and amastatin was assayed in continuous rate assays.

serine, cysteine and aspartic acid proteases (Table II). Chelating agents such as EDTA and 1,10-phenanthroline abolished activity. Inhibition by EDTA was time dependent. The enzyme was completely inhibited after incubation in 10 mM EDTA in Tris-HCl, pH 7.2 at 4°C for 5 h. Besides chelating agents, bestatin and amastatin are both strong inhibitors of aminopeptidase yscXVI.

3.3. *Influence of metals*

The activity of aminopeptidase yscXVI was not affected by 1 mM $ZnCl_2$. More than two-fold activation of the purified enzyme was achieved with $CoCl_2$ (Table III). After inhibition by EDTA and dialysis against 25 mM Tris-HCl, pH 7.2, enzyme activity was restored by Mn^{2+}, Zn^{2+} and Co^{2+} ions (Table III).

3.4. *Kinetic studies*

For the hydrolysis of (Cys-NH-Ph)$_2$ the K_m was 22.5 μM, and V_{max} was 12.9 mU/mg. K_i values of 0.54 μM and 0.93 μM were calculated for the inhibition of the enzyme by bestatin and amastatin, respectively.

Table III

Hydrolysis of (Cys-NH-Ph)$_2$ was measured in the presence and absence of metal ions

Treatment	Relative hydrolysis rate
None	100
None + 300 μM $CoCl_2$	220
None + 1 mM $ZnCl_2$	101
EDTA	4
EDTA, dialysis + 100 μM $MnCl_2$	114
EDTA, dialysis + 100 μM $ZnCl_2$	79
EDTA, dialysis + 100 μM $CoCl_2$	118

When indicated aminopeptidase yscXVI was treated with 10 mM EDTA for 24 h at 4°C, and dialysed against 25 mM Tris-HCl, pH 7.2. Activity of the dialysed enzyme was assayed in the presence of 10 μM EDTA and various metals.

3.5. *Substrate specificity*

Besides (Cys-NH-Ph)$_2$ aminopeptidase yscXVI hydrolyzed several other unblocked 4-nitroanilide substrates (Table IV). To test whether the enzyme has endoproteolytic activity aminopeptidase yscXVI was incubated together with aminopeptidase M (Boehringer-Mannheim) and a series of N-terminal blocked substrates. No degradation of the following peptide derivatives was observed: Suc-Ala-Ala-Pro-Phe-NH-Ph, Suc-Ala-Ala-Pro-Leu-NH-Ph, Suc-Ala-Ala-Val-Ala-NH-Ph, Z-Arg-Arg-NH-Ph, Z-Gly-Pro-NH-Ph, Z-Pro-Phe-Arg-NH-Ph, Ac-Arg-Gly-Gly-Met-NH-Ph, Bz-Ile-Glu-Gly-Arg-NH-Ph, Bz-Tyr-NH-Ph, Z-Lys-Arg-NH-Ph.

3.6. *Enzyme activity under various growth conditions, in a different strain of yeast, and in the vacuole*

Yeast cells were harvested in the logarithmic growth phase (A_{600} 0.8), in the stationary phase (A_{600} 13) and after starvation of a stationary culture in 1% potassium acetate at 30°C for 6 h. The specific activities in the crude extract of these cultures were 0.112, 0.077 and 0.115 mU/mg protein, respectively. In a stationary culture of a yeast strain lacking aminopeptidase yscII (YHH26) we measured the enzyme activity as 0.272 mU/mg protein.

In vacuoles we detected less than 2% of the (Cys-NH-Ph)$_2$ degrading activity present in the lysate. Aminopeptidase yscXVI is therefore considered to be non-vacuolar.

4. DISCUSSION

In the present study aminopeptidase yscXVI was purified from *Saccharomyces cerevisiae*. Although in yeast it is not uncommon to lose half of the activity during

Table IV

Hydrolysis of various 4-nitroanilide substrate by aminopeptidase yscXVI

Substrate	Relative hydrolysis rate
(Cys-NH-Ph)$_2$	100
Lys-NH-Ph	676
Leu-NH-Ph	544
Arg-NH-Ph	500
Met-NH-Ph	255
Phe-NH-Ph	215
Ala-NH-Ph	196
S-Bz-Cys-NH-Ph	109
Val-NH-Ph	67
Glu-NH-Ph	58
Gly-NH-Ph	47
Pro-NH-Ph	31

The final substrate concentration was 100 μM in all assays.

the first purification step [16], the early and late eluting form of the enzyme that eluted from DEAE-Sephacel represent together only one third of the activity measurable in the crude extract of yeast cells. This very low recovery seems to be due partly to some very strong binding of a cystinyl bond hydrolyzing activity to the ion-exchange resin, and partly to some irreversible inactivation of aminopeptidase yscXVI during purification. After elution of the DEAE-Sephacel column with 300 mM NaCl some enzyme activity was still detectable in the ion-exchange resin. This activity could not be eluted with salt concentrations up to 1 M. Addition of detergents or 10% glycerol to the elution buffer did not improve purification. To find out whether aminopeptidase yscXVI was separated from some essential cofactors during the first purification step, crude yeast extract was added to the fractions, but only a minimal increase in enzyme activity was observed.

Due to inhibition by chelating agents and reactivation by metal ions, aminopeptidase yscXVI is classified as a metallo-enzyme. This seems to be the most common class for yeast aminopeptidases. Results of inhibitor studies were reported for 12 yeast aminopeptidases, and all except aminopeptidase LAP III were inhibited by EDTA [19,21,28]. In contrast to other metallo-peptidases, the activity of aminopeptidase yscXVI was not decreased by a high concentration of Zn^{2+} [21]. Like all of the previously tested yeast aminopeptidases [21], except aminopeptidase yscCo [29], aminopeptidase yscXVI is inhibited by bestatin, an inhibitor known to abolish the activity of many mammalian aminopeptidases [35]. Amastatin, a rather specific inhibitor of mammalian glutamyl aminopeptidase and leucyl aminopeptidase [35], is also a strong inhibitor of aminopeptidase yscXVI. Whether amastatin affects other yeast aminopeptidases has not yet been tested.

Like other aminopeptidases of mammalian and yeast origin aminopeptidase yscXVI cleaves various unsubstituted N-terminal L-amino acids of peptide substrates. $(Cys\text{-}NH\text{-}Ph)_2$ was used to monitor progress of purification because, in a previous study [30] with this substrate, only two peaks of activity were detected in the eluate of ion-exchange chromatography, whereas Leu-NH-Ph and Lys-NH-Ph are hydrolyzed by several aminopeptidases [21]. It seems that the cleavage of an N-terminal cysteine residue is only catalysed by a few aminopeptidases. Mammalian leucyl aminopeptidase, an enzyme frequently used in biochemical research, also does not act on substrates with an N-terminal cysteine residue [35].

Aminopeptidase yscXVI was detected in a yeast strain deficient in aminopeptidase yscII. Therefore identity with this aminopeptidase is excluded. By comparing the characteristics of aminopeptidase yscXVI with the data of other yeast aminopeptidases we could not correlate the newly discovered aminopeptidase yscXVI to any one of the previously described enzymes.

Aminopeptidase yscXVI shares some similarity with the mammalian cystinyl aminopeptidase (EC 3.4.11.3), also known as oxytocinase [35,36]: both enzymes are metallo-peptidases and cleave the peptide bond between an N-terminal cysteine and the adjacent residue. In contrast to aminopeptidase yscXVI, cystinyl aminopeptidase is insensitive to bestatin and, even more important, like most mammalian aminopeptidases, cystinyl aminopeptidase has a molecular weight over 100,000 Da. This excludes a relationship between the two enzymes. Similarities with other mammalian aminopeptidases were not discovered after studying the literature of mammalian aminopeptidases.

The function of aminopeptidase yscXVI is unknown. It is also not understood why such a high number of aminopeptidases is present in the yeast *Saccharomyces cerevisiae*. Several of these aminopeptidases have overlapping substrate specificities [1–4,22,37]. It is therefore not surprising that mutations in up to four aminopeptidases [28], or even deletion of a gene encoding an aminopeptidase [27] has only little influence on the growth of the yeast.

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